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Reclassification of [*Cytophaga*] *marinoflava* Reichenbach 1989 as *Leeuwenhoekiella marinoflava* gen. nov., comb. nov. and description of *Leeuwenhoekiella aequorea* sp. nov.

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Five heterotrophic, aerobic, halotolerant and pigmented bacterial strains with gliding motility were isolated from Antarctic sea water; one other isolate was collected from the sea urchin *Strongylocentrotus intermedius* in the Gulf of Peter the Great in the Sea of Japan. 16S rRNA gene sequence analysis indicated that the strains are members of the family *Flavobacteriaceae*, the nearest neighbour (with 97·1 % sequence similarity) being the misclassified species [*Cytophaga*] *marinoflava*. DNA–DNA hybridization experiments and chemotaxonomic and phenotypic analyses demonstrated that the six novel isolates represent a single species distinct from [*C.*] *marinoflava*. On the basis of its separate phylogenetic lineage (the nearest neighbours show 92 % sequence similarity), [*C.*] *marinoflava* is reclassified as *Leeuwenhoekiella marinoflava* gen. nov., comb. nov. A second species of this new genus, *Leeuwenhoekiella aequorea* sp. nov., is proposed for the six novel isolates, with strain LMG 22550^T (=CCUG 50091^T) as the type strain.

Many novel taxa that inhabit shallow-water environments have been described within the family *Flavobacteriaceae* in recent years (Bernardet *et al.*, 2002). Most coastal-water flavobacteria, e.g. members of the genera *Arenibacter*, *Cellulophaga*, *Gelidibacter*, *Mesonia*, *Muricauda*, *Polaribacter*, *Psychroserpens*, *Tenacibaculum*, *Ulvibacter*, *Vitellibacter* and *Zobellia*, require NaCl or sea water for growth and are described as weakly or moderately halophilic (Kushner, 1978; Reichenbach, 1989; Bowman *et al.*, 1997; Gosink *et al.*, 1998; Johansen *et al.*, 1999; Barbeyron *et al.*, 2001; Bruns *et al.*, 2001; Ivanova *et al.*, 2001; Suzuki *et al.*, 2001; Nedashkovskaya *et al.*, 2003b, d, 2004a). The halotolerant taxa of the family *Flavobacteriaceae*, e.g. *Salegentibacter salegens* and *Psychroflexus gondwanensis*, can grow without Na^+ ions or sea water and can tolerate high salinity levels (Dobson *et al.*, 1993; McCammon & Bowman, 2000; Bowman *et al.*, 1998).

The genus *Cytophaga* was established by Winogradsky (1929) and emended by Reichenbach (1989). Later, Nakagawa & Yamasato (1996) proposed the restriction of this genus on the basis of 16S rRNA gene sequence phylogenetic analysis, and emended the genus description. Currently, the genus *Cytophaga sensu stricto* (aerobic, gliding, pigmented, cellulose-degrading bacteria) comprises two species: *Cytophaga aurantiaca* and *Cytophaga hutchinsonii*. Some marine bacteria previously included in the genus *Cytophaga* have been reclassified (Nakagawa & Yamasato, 1996; Nakagawa *et al.*, 1997; Johansen *et al.*, 1999; Suzuki *et al.*, 2001; Barbeyron *et al.*, 2001; Nedashkovskaya *et al.*, 2005). At present, two misnamed species of the genus *Cytophaga* that were isolated from marine environments, [*Cytophaga*] *fermentans* and [*Cytophaga*] *marinoflava*, remain to be reclassified.

In this work, we report the isolation and identification of six novel halotolerant, Gram-negative, aerobic, gliding,

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yellow-pigmented marine bacteria. On the basis of the results of genotypic, chemotaxonomic and phenotypic analyses, it is clear that the isolates represent a novel species, with [*C.*] *marinoflava* as the nearest neighbour. Both taxa are here classified in a single novel genus, as *Leeuwenhoekiella marinoflava* gen. nov., comb. nov. and *Leeuwenhoekiella aequorea* sp. nov.

Strains LMG 22550^T (=ANT 14^T), LMG 22551 (=ANT 18d/2), LMG 22552 (=ANT 26b), LMG 22553 (=ANT 35/2) and LMG 22554 (=ANT 54b/2) were isolated previously from Antarctic sea-water samples at stations above Gunnerus Ridge and Astrid Ridge (Tan *et al.*, 1999), using enrichment in dialysis chambers (Tan, 1997). Strain KMM 6066 (=LMG 22555) was isolated from the sea urchin *Strongylocentrotus intermedius* in Troitsa Bay, Gulf of Peter the Great, Sea of Japan. For the isolation of the latter strain, 0·1 ml tissue homogenate was transferred onto plates of marine agar 2216 (Difco). After primary isolation and purification, strains were cultivated at 28 °C on the same medium and stored at −80 °C in marine broth 2216 (Difco) supplemented with 20 % (v/v) glycerol.

An almost-complete 16S rRNA gene sequence (1475 nt) of one representative of the Antarctic isolates, strain LMG 22550^T, was determined previously in a study on the diversity of facultative oligotrophic bacteria from polar seas (Mergaert *et al.*, 2001). The sequence of strain KMM 6066 (1474 nt) was determined in the present study by following a procedure described previously (Vancanneyt *et al.*, 2004) and showed a similarity of 99·8 % with respect to LMG 22550^T. The nearest phylogenetic neighbour of both strains was [*C.*] *marinoflava* ATCC 19326^T (=LMG 1345^T): the 16S rRNA gene sequence similarity was 97·1 %. The three strains formed a distinct lineage within the family *Flavobacteriaceae*, showing sequence similarity levels below 92·2 % with the genera *Vitellibacter*, *Aequorivita*, *Arenibacter*, *Muricauda*, *Zobellia* and *Maribacter* (Fig. 1). These observations allow reclassification of members of the [*C.*] *marinoflava* branch within a novel genus.

DNA G+C contents were determined for strains LMG 22550^T to LMG 22555 and for [*C.*] *marinoflava* LMG 1345^T. Strains were cultivated on marine agar 2216 for 24 h at 37 °C. DNA was extracted from 0·75–1·25 g (wet wt) cells, using the DNA extraction protocol of Wilson (1987) as modified by Cleenwerck *et al.* (2002). Cells were lysed in a Tris/EDTA buffer (10 mM Tris/HCl with up to 200 mM EDTA, pH 8·0) containing RNase A (Sigma), SDS (Serva) and proteinase K (Merck) to final concentrations of 400 μg ml^{-1}, 2 % (w/v) and 200 μg ml^{-1}, respectively. NaCl (5 M stock solution) and CTAB/NaCl solution (10 %, w/v, hexadecyltrimethylammonium bromide in 0·7 M NaCl) were added to final concentrations of 1 M and 13·3 % (v/v), respectively. For determination of G+C content, DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated by HPLC using a Waters Symmetry Shield C8 column maintained at a temperature of 37 °C. The solvent was 0·02 M $NH_4H_2PO_4$ (pH 4·0) with 1·5 % acetonitrile. Non-methylated phage λ DNA (Sigma) was used as the calibration reference.

The DNA G+C contents were 35–36 mol% for the novel isolates (LMG 22550^T to LMG 22555) and 38 mol% for the type strain of [*C.*] *marinoflava* (LMG 1345^T). DNA–DNA hybridizations were performed between strains LMG 22550^T to LMG 22555 and [*C.*] *marinoflava* LMG 1345^T with DNA prepared as described above. The microplate method was used as described by Ezaki *et al.* (1989) and Goris *et al.* (1998), using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. Biotinylated DNA was hybridized with single-stranded unlabelled DNA, non-covalently bound to microplate wells. Hybridizations were performed at 36 °C in a hybridization mixture [2 × SSC, 5 × Denhardt's solution, 2·5 % dextran sulphate, 50 % formamide, 100 μg denatured low-molecular-mass salmon sperm DNA ml^{-1}, 1250 ng biotinylated probe DNA ml^{-1}]. Hybridization levels of 79–100 % were found between strains LMG 22550^T to LMG

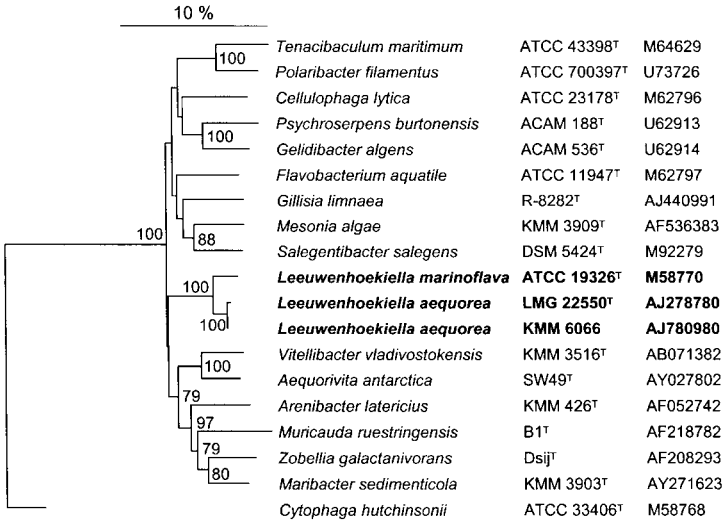

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequences of strains ATCC 19326^T and LMG 22550^T and representative members of related genera in the family *Flavobacteriaceae*. The tree was generated by using the neighbour-joining method (Saitou & Nei, 1987). The numbers at nodes indicate bootstrap values (%). Bar, 0·1 substitutions per nucleotide position.

22555, which indicates that the strains constitute a single species. The latter strains had binding values of 9–14 % with [*C.*] *marinoflava* LMG 1345^T. These data indicate that the novel isolates constitute a single species distinct from the latter misclassified species (Wayne *et al.*, 1987).

The analysis of fatty acid methyl esters was carried out according to the standard protocol of the Microbial Identification System (Microbial ID). The main cellular fatty acids of the strains studied were 15 : 0 iso, 15 : 1 iso G, 17 : 0 iso 3-OH, iso 17 : 1ω9*c* and summed feature 3 (see Table 1). [*C.*] *marinoflava* is distinguished from the novel isolates by a significantly larger amount of 17 : 0 iso 3-OH and smaller amount of iso 17 : 1ω9*c*. Furthermore, the presence of minor amounts of 15 : 0 3-OH and the absence of anteiso 17 : 1ω9*c* and summed feature 4 (Table 1) fatty acids in the [*C.*] *marinoflava* whole-cell fatty acid profile support its differentiation from strains LMG 22550^T to LMG 22555. Isoprenoid quinones were extracted from lyophilized cells and analysed as described previously (Nedashkovskaya *et al.*, 2003d). The major respiratory quinone was MK-6.

Table 1. Fatty acid content (mean percentage of total) of members of the genus *Leeuwenhoekiella* gen. nov.

Those fatty acids for which the amount (for all taxa) is less than 1 % are not given. tr, Trace amount (less than 1 %); *n*, number of strains studied.

Fatty acid	*L. aequorea* (*n*=6)	*L. marinoflava* LMG 1345^T
15 : 0 2-OH	1·2±0·2	1·4
15 : 0 3-OH	–	1·3
15 : 0 anteiso	4·5±0·4	2·7
15 : 0 iso	18·2±1·1	16·0
15 : 1 iso G	7·6±1·6	10·4
15 : 0 iso 3-OH	2·1±0·2	3·9
16 : 0 iso	3·8±1·2	1·1
16 : 1 iso H	1·4±0·4	tr
16 : 0 iso 3-OH	3·4±0·9	2·5
17 : 0 2-OH	4·2±0·6	4·4
17 : 0 iso 3-OH	12·7±1·0	22·1
17 : 1ω6*c*	2·1±0·4	1·8
17 : 1ω8*c*	tr	1·3
anteiso 17 : 1ω9*c*	1·6±0·4	–
iso 17 : 1ω9*c*	18·8±2·9	12·1
Summed feature 3*	9·4±1·6	10·9
Summed feature 4*	1·3±0·2	–
ECL 13·565†	tr	1·3
ECL 16·582†	tr	1·2

*Summed features consist of one or more fatty acids that could not be separated by the Microbial Identification System. Summed feature 3: 15 : 0 iso 2-OH, 16 : 1ω7*c* and/or 16 : 1ω7*t*. Summed feature 4: 17 : 1 iso I and/or 17 : 1 anteiso B.

†Unidentified fatty acids (ECL, equivalent chain length).

Phenotypic analysis was performed by using previously described methods (Nedashkovskaya *et al.*, 2003a, b). The physiological, biochemical and morphological characteristics of the strains studied are listed in the species description and in Table 2. The results of phenotypic examination of the strains studied, including the ability of strains LMG 22550^T to LMG 22555 to form acid from D-sucrose and mannitol and to utilize mannitol, in combination with the molecular distinctiveness allow the differentiation of strains LMG 22550^T to LMG 22555 from their closest relative, [*C.*] *marinoflava*, at the species level. However, on the basis of the phenotypic analysis, the cellular fatty acid composition and the phylogenetic positions of the strains examined, we propose that LMG 22550^T to LMG 22555 and [*C.*] *marinoflava* LMG 1345^T represent two distinct species of the same novel genus. The main phenotypic characteristics that differentiate the strains studied from other relatives in the family *Flavobacteriaceae* are listed in Table 2.

We conclude that the bacteria studied could not be assigned to any of the existing genera or species currently included in the family *Flavobacteriaceae*. Consequently, we propose that strains LMG 22550^T to LMG 22555 be placed in a novel genus, *Leeuwenhoekiella* gen. nov., as *Leeuwenhoekiella aequorea* sp. nov., and that [*C.*] *marinoflava* be reclassified as *Leeuwenhoekiella marinoflava* comb. nov.

Description of *Leeuwenhoekiella* gen. nov.

Leeuwenhoekiella [Leeu.wen.hoe.ki.el′la. N.L. fem. dim. n. *Leeuwenhoekiella* of Leeuwenhoek, named in honour of the famous Dutchman Antonie van Leeuwenhoek (1632–1723), discoverer of micro-organisms].

Rod-shaped cells, motile by gliding. Gram-negative. Endospores are not formed. Strictly aerobic. Produces non-diffusible yellow pigments. No flexirubins are formed. Chemo-organotrophic. Cytochrome oxidase-, catalase-, β-galactosidase- and alkaline phosphatase-positive. The major respiratory quinone is MK-6. The dominant cellular fatty acids (>5 %) are 15 : 0 iso, 15 : 1 iso G, 17 : 0 iso 3-OH, iso 17 : 1ω9*c* and summed feature 3 (see Table 1). According to 16S rRNA gene sequence phylogenetic analysis, the genus *Leeuwenhoekiella* is a member of the family *Flavobacteriaceae*. The type species is *Leeuwenhoekiella marinoflava*.

Description of *Leeuwenhoekiella marinoflava* comb. nov.

Leeuwenhoekiella marinoflava (ma.ri.no.fla′va. L. adj. *marinus* marine; L. adj. *flavus* golden yellow; N.L. fem. adj. *marinoflava* marine and yellow-pigmented).

Basonym: *Cytophaga marinoflava* (*ex* Colwell *et al.* 1966) Reichenbach 1989.

The main characteristics are those as given for the genus and by Reichenbach (1989). In addition, growth is observed at 4–37 °C. Optimal temperature for growth is 21–23 °C.

Table 2. Differential characteristics of the genus *Leeuwenhoekiella* and other related genera of the family *Flavobacteriaceae*

Genera: 1, *Leeuwenhoekiella* gen. nov.; 2, *Arenibacter*; 3, *Zobellia*; 4, *Muricauda*; 5, *Vitellibacter*; 6, *Aequorivita*; 7, *Maribacter*. Data were taken from Barbeyron *et al.* (2001), Bruns *et al.* (2001), Ivanova *et al.* (2001), Bowman & Nichols (2002), Nedashkovskaya *et al.* (2003b, c, 2004b, c) and this study. Abbreviations: −, negative; +, positive; V, variable; ND, not determined.

Characteristic	1	2	3	4	5	6	7
Anaerobic growth	−	−	−	+	−	−	−
Gliding motility	+	−	+	+	−	−	+
Oxidase/catalase	+/+	+/+	+/+	+/−	+/+	−/+	+/+
Flexirubin pigments	−	−	+	−	+	−	−
Requirement for Na^+ for growth	−	+	+	+	+	V	+
Acid formation from carbohydrates	+	+	+	ND	−	−	V
Growth at/in:							
37 °C	+	+	V	+	+	−	−
42 °C	−	V	V	−	+	−	−
15 % NaCl	+	−	−	−	−	−	−
Hydrolysis of:							
Agar	−	−	+	−	−	−	V
Casein	+	−	V	ND	+	V	−
Gelatin	+	V	+	−	+	+	V
Starch	+	−	V	−	−	−	V
DNA	−	V	V	ND	+	−	V
Nitrate reduction	−	+	+	−	−	−	V
DNA G+C content (mol%)	35–38	37–40	36–44	41	41	33–39	35–39

Growth occurs at 0–15 % NaCl, with optimal growth at 1–3 % NaCl. Nitrate is not reduced. Indole, H_2S and acetoin (Voges–Proskauer reaction) are not produced. Decomposes casein, gelatin, Tweens 20, 40 and 80 and starch. Does not hydrolyse DNA, urea, cellulose (CM-cellulose and filter paper) or chitin. Forms acid from D-galactose and glycerol, but not from L-arabinose, D-cellobiose, L-fucose, D-glucose, D-lactose, D-maltose, D-melibiose, L-raffinose, L-rhamnose, L-sorbose, D-sucrose, D-trehalose, DL-xylose, *N*-acetylglucosamine, citrate, acetate, fumarate, malate, adonitol, dulcitol, inositol or mannitol. Utilizes L-arabinose, D-glucose, D-lactose, D-mannose and D-sucrose, but not inositol, sorbitol, mannitol, malonate or citrate. Susceptible to benzylpenicillin, carbenicillin, lincomycin, doxycycline, erythromycin and chloramphenicol. The G+C content of the DNA is 38 mol%.

The type strain is LMG 1345^T (=ATCC 19326^T). Isolated from sea water collected in the North Sea off Aberdeen, Scotland, UK.

Description of *Leeuwenhoekiella aequorea* sp. nov.

Leeuwenhoekiella aequorea (ae.quo.re′a. L. fem. adj. *aequorea* of the sea, marine).

The main characteristics are as given for the genus. In addition, cells range from 0·5 to 0·6 μm in width and from 1·6 to 2·3 μm in length. On marine agar 2216, colonies are 2–4 mm in diameter, circular with entire edges and bright yellow in colour. Growth is observed at 4–37 °C. Optimal temperature for growth is 23–25 °C. Growth occurs at 0–15 % NaCl, with optimal growth at 0–5 % NaCl. Nitrate is not reduced. Indole, H_2S and acetoin (Voges–Proskauer reaction) are not produced. Decomposes casein, gelatin, starch and Tweens 20, 40 and 80. Does not hydrolyse agar, DNA, urea, cellulose (CM-cellulose and filter paper) or chitin. Forms acid from D-galactose, D-sucrose, glycerol and mannitol, but not from L-arabinose, D-cellobiose, L-fucose, D-glucose, D-lactose, D-maltose, D-melibiose, L-raffinose, L-rhamnose, L-sorbose, DL-xylose, *N*-acetylglucosamine, acetate, citrate, fumarate, malate, adonitol, dulcitol or inositol. Can oxidize D-trehalose. Utilizes L-arabinose, D-glucose, D-lactose, D-mannose, D-sucrose and mannitol, but not inositol, sorbitol, malonate or citrate. The G+C content of the DNA is 35–36 mol%.

The type strain is LMG 22550^T (=CCUG 50091^T), which was isolated from Antarctic sea water. Strain LMG 22555 was isolated from the sea urchin *Strongylocentrotus intermedius* found in the Sea of Japan.

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